THE EFFECT OF VARIOUS CONCENTRATIONS OF GOSSYPOL ON THE RUMEN PROTOZOAL DEGRADATION OF GRASS HAY IN CULTURE

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ABSTRACT

The aim of this experiment was to examine the effects of gossypol on rumen protozoal fermentation of grass hay (GH) with and without 1mM, 5mM, and 10mM gossypol in culture. Gas production from GM (*in vitro*) was measured according to the method of Menke and Steingass (1998). The rate of gas production was determined by fitting cumulative gas production data on an exponential equation $y = a + b\ (1-e^{-ct})$. Dry matter (DM) losses were measured after 24 h incubation. Microscopic observation of the supernatant from syringes was conducted to examine the death and the surviving rumen cilliates. The supernatant was also analysed by using HPLC for the concentration of VFA in culture. Statistical analysis based on randomised complete design (RCD) was employed to examine the relative DM losses and Volatile Fatty Acids (VFA) concentrations between the treatment. The results of this experiment shows that the rate of gas production (c) from GH is higher than that of GH treated with gossypol. However, the potential gas production (a + b) and the asymptote (b) of GH were much lower than gossypol treated substrates suggesting that the presence of gossypol in the culture resulted in more gas from GH. The reason for this is uncertain. However, the increase in gas production which occurred when gossypol was added to GH conversely correlated with the DM losses of the GH. Microscopic observation of the rumen fluid from syringes indicated that syringe containing the GH substrate has a very active population of rumen ciliates with very few dead organism. In contrast gossypol caused the death of up 75% rumen protozoa in culture. In the presence of gossypol, there was a change in the proportions of VFA suggesting that the presence of gossypol may have altered the fermentative activity of rumen protozoa in culture. It was concluded that gossypol was responsible for the death of about 75% rumen cilliates which reduced the fermentation of GH in culture. However, gas production increased when the death of the rumen protozoa increased.

(Key words : Gossypol, Rumen Protozoa, Grass Hay, Gas production).

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PENGARUH GOSSYPOL TERHADAP DEGRADASI DAN FERMENTASI HAY OLEH PROTOZOA RUMEN

INTISARI

Tujuan dari penelitian ini adalah untuk menguji kemampuan protozoa rumen dalam memfermentasi hay dengan atau tanpa adanya antinutrien gossypol (1 mM, 5mM, dan 10mM) dalam kultur. Penelitian ini menggunakan metode 'produksi gas' (*in vitro)* sebagaimana dijelaskan oleh Menke dan Steingass (1988). Produksi gas dicatat setelah 2, 4, 6, 8, 10, 12, 22 dan 24 jam inkubasi. Data produksi gas dimasukkan kedalam persaman $y = a + b\ (1\text{-}e^{-ct})$ untuk menentukan kurva produksi gas. Pemeriksaan supernatan secara mikroskopis dilakukan untuk melihat protozoa yang hidup dan yang mati setelah 24 jam inkubasi, dan sebagian supernatan dianalisis kandungan asam lemak terbang. Kemudian bahan kering tercerna diukur setelah dilakukan pencucian dan pengeringan sisa supernatan dalam syringe. Analisis statistik berdasarkan rancangan acak lengkap (RAL) dilakukan untuk melihat perbedaaan bahan kering tercerna dan konsetrasi asam lemak terbang antara perlakuan satu dengan lainnya. Hasil penelitian ini menunjukkan bahwa kecepatan produksi gas (c) dari hay lebih tinggi dibandingkan dengan hay yang diberi gossypol. Namun demikian potensial produksi gas (a+b) dari hay yang ditambah gossypol lebih tinggi dibanding dengan perlakuan kontrol hay. Hal ini menunjukkan bahwa gossypol dalam kultur tersebut menyebabkan peningkatan produksi gas. Kenaikan produksi gas tersebut berkorelasi negatif terhadap kecernaan bahan kering subtrat hay. Hasil pemeriksaan mikroskopis menunjukkan bahwa gossypol menyebabkan kematian sekitar 75% protozoa rumen. Gossypol juga mengubah molar proporsi asam lemak terbang, yang menunjukkan bahwa gossypol tersebut mempengaruhi aktivitas fermentasi pakan hay oleh protozoa rumen. Kesimpulan dari penelitian adalah bahwa gossypol bertanggung jawab atas kematian sebagian besar protozoa yang menyebabkan rendahnya bahan kering tercerna dan perubahan aktivitas fermentasi hay, sedangkan produksi gas semakin meningkat sejalan dengan meningkatnya jumlah protozoa yang mati.

(Kata kunci : Gossypol, Protozoa rumen, Hay, Produksi gas).

Introduction

The previous experiment (Ismartoyo *et al*, 1993; 1994; 1995) demonstrated that the degradation and fermentation activities of mixed rumen microorganisms and of rumen bacteria were reduced by the presence of gossypol in the continuous batch culture system. In contrast to the situation with the mixed microbial flora there was no evidence for adaptation of rumen bacteria to gossypol. A further experiment was conducted to examine the effect of various concentrations of gossypol on the degradation of GH by rumen protozoa. The objectives of this experiment were:

1. To prepare a fraction rich in rumen protozoa from whole rumen fluid.
2. To incubate these protozoa *in vitro* and to measure gas production at 2, 4, 6, 8, 10, 12, 22 and 24 h of incubation.
3. To measure DM loss of GH after 24 h of incubation.
4. To measure VFA production from GH by protozoa after 24 h of incubation.
5. To measure rate of gas production by fitting cumulative gas production data on an exponential equation $y = a + b\ (1\text{-}e^{-ct})$ described by Ørskov and McDonald (1979). Where 'y' was cumulative gas production at time 't', (a + b) was potential

gas production, and 'c' was the rate of gas production; a, b and c are constants.

Materials and Methods

Sample preparation

Samples of grass hay (GH) were milled using a screen of 1 mm. Samples of GH (200 mg) with and without gossypol (Risco *et al*, 1997) were weighed accurately and transferred to duplicate syringes (100 ml, with capillary attachment and a clip) and piston, greased with vaseline, was pushed into the barrel.

Rumen fluid preparation

Whole rumen fluid were collected from sheep fitted with a permanent cannula and fed GP (general purpose) diet, which consisted of hay 500 g kg^{-1}, barley 300 g kg^{-1}, molasses 100 g kg^{-1}, fish meal 91 g kg^{-1}, salt 6 g kg^{-1}, mixed minerals and vitamins 1 g kg^{-1}. The minerals and vitamins consisted of calcium 185 g kg^{-1}, magnesium 104 g kg^{-1}, cobalt 2.25 g kg^{-1}, manganese 44 x 10^3 mg kg^{-1}, zinc 36.4 g kg^{-1}, iodine 1300 mg kg^{-1}, selenium 100 mg kg^{-1}, vitamin A 10^6 IU kg^{-1}, vitamin D3 2 x 10^6 IU kg^{-1} and vitamin E 4 x 10^3 IU kg^{-1}.

Fractionation of rumen protozoa

The separation of rumen protozoa from bacteria was carried out as described by Eadie and Oxford (1955). Whole rumen fluid was screened through a double layer of muslin to remove larger particles and debris. The filtrate was transferred immediately to separating funnels (250 ml) and incubated at 39°C for 30 min leading to the deposition of protozoa as a bottom layer. This white bottom layer was withdrawn into test tubes nearly filled with the buffer solution. A subsample was taken for light microscopic observation of the rumen protozoa.

Preparation of media solution

Medium solution (Menke and Steingass, 1988) was prepared at room temperature including, firstly, the major element solution which consisted of 5.7 g Na_2HPO_4, 6.2 g $KHPO_4$, 0.6 g $MgSO_4.7H_2O$ and was made up to 1 litre with distilled H_2O. Secondly, 1 litre buffer solution was made up consisting of 35 g $NaHCO_3$, 4 g $(NH_4)HCO_3$ and distilled H_2O. Thirdly, 1 litre of trace element solution consisting of 13.2 g $CaCl_2.2H_2O$, 10 g $MnCl_2.4H_2O$, 1.0 g $CoCl_2.6H_2O$, 0.8 g $FeCl_2.6H_2O$ and distilled H_2O.

A reducing solution was made up of 2 ml 1-M-NaOH, 285 mg $Na_2S.7H_2O$ and 47.5 ml distilled H_2O. This reducing solution was freshly prepared each time shortly before rumen fluid was taken. The solutions were poured into an Erlenmeyer flask (1 l), mixed in the following order: 474 ml distilled H_2O, 0.12 ml trace element solution, 237 ml buffer solution, 237 ml major element solution and 1.22 ml resazurin solution, and heated to 39°C in water bath. O_2-free CO_2 was bubbled into the mixture while the reducing solution was being added. The slightly bluish solution first turned red and then became colourless, indicating from the reduction of the indicator resazurin that the solution was reduced (free from O_2).

Determination of gas production

The rumen protozoa fluid-buffer mixture (1: 2) was dispensed in 30 ml amounts using a pipette (50 ml) into glass syringes (100 ml) containing 200 mg DM substrate. The tube on the capillary attachment at the bottom of the syringe (see Figure 4) was firmly fixed to the pipette. Air bubbles were brought to the surface by gentle shaking and removed through the capillary attachment by a careful upward movement of the piston. After shutting the clip on the tube the exact volume was read off, and the syringe was placed in the water bath which had been pre-warmed to 39°C. The displacement of the piston by gas was read at 2, 4, 6, 8, 10, 22, and 24 h after inoculation with protozoa. Duplicate syringes containing buffer and protozoa suspension (without GH substrate) were prepared for blanks and treated in the same manner with other treatments.

After 24 h of incubation 2 ml suspension was withdrawn by pipette from each syringe and the rumen protozoa were examined under a microscope. Whole suspension containing sample and buffer mixture were transferred into tubes (50 ml) and were centrifuged at room temperature (20°C) at 200*g for 10 min. The feed residues were then freeze dried for determination of DM losses. Supernatant were stored at –20°C prior to VFA analysis.

Analysis of 'Menke' gas production data

The volumes of gas at each reading were used to calculate the cumulative gas production over 24 h of incubation. The cumulative gas production were corrected with the mean of cumulative gas production from the blank and then converted to cumulative gas production per 200 mg dry matter. Data of gas production for each sample was fitted to an exponential equation $y = a + b (1-e^{-ct})$, using a NOWAY program for calculation of degradation curves as described by Harbron (1994, SASS, Rowett Research Institute, Bucksburn, Aberdeen).

Results

To examine the effects of various concentrations of gossypol (1, 5, and 10 mM) on gas production from grass hay (GH) with and without gossypol, these substrates were incubated at 39°C for 24 h. Gas production was recorded at 2, 4, 6, 8, 10, 12, 22 and 24 h of incubation. The results of this experiment are shown in Table 1.

Table 1 and Figure 1 show that gas production from fermentation of GH (without gossypol) at the end of 24 h incubation period was significantly ($P<0.01$) lower than those of GH treated with gossypol. In the presence of gossypol, gas production from GH was significantly ($P<0.01$) decreased as the gossypol concentration increased. To examine the effect of gossypol on the rumen protozoal fermentation of GH, the data were fitted with the exponential equation $p = a + b (1-e^{-ct})$ and the results of this calculation are shown in Table 2.

Table 1. Gas production (cm^3 200 mg^{-1} DM) from grass hay with and without gossypol when incubated with rumen protozoa (n = 2)

Time of incubation (h)	Substrates			
	GH	GH+1mM gossypol	GH+5mM gossypol	GH+10mM gossypol
2	1.7	3.0	3	2.7
4	4.0	4.7	6	5.2
6	5.2	7.5	8.5	7.2
8	8.0	11.0	10.5	8.5
10	9.0	14.2	12.7	8.2
12	11.5	18.1	15.6	14.1
22	13.5	31.7	29.0	24.0
24 (Final)	15.7 a	32 b	28 c	22.5 d

Values with different subscripts are significantly different ($P<0.01$, SED = 0.10).

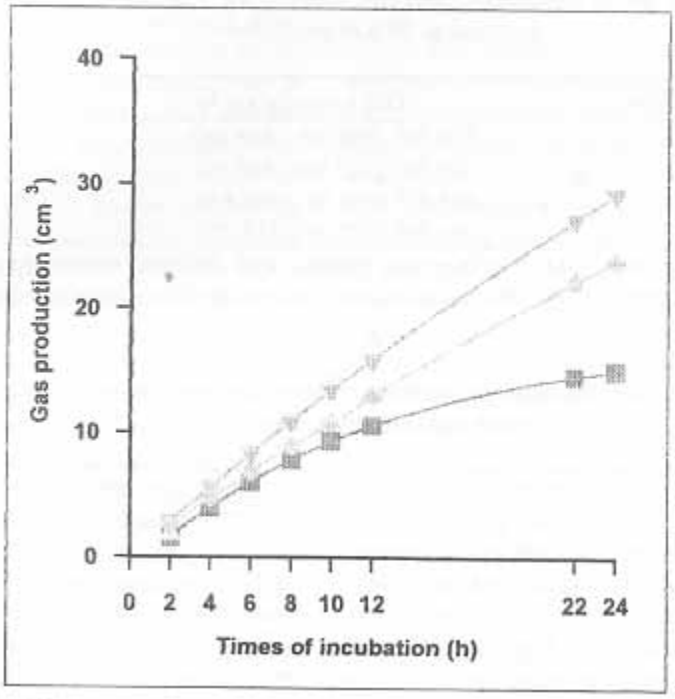

Figure 1. Gas production from grass hay with and without gossypol incubated with fraction of rumen content rich in rumen protozoa *in vitro* fitted to an exponential equation of $y = a + b (1 - e^{-ct})$.

Table 2. Degradation characteristics from fermentation of GH with and without gossypol by rumen protozoa

Substrates	a	b	c	(a + b)	RSD
GH	-1.3	19.1	0.081	17.7	0.81
GH + 1 mM gossypol	-1.3	123.5	0.013	122.3	1.03
GH + 5 mM gossypol	0.4	144.4	0.009	144.4	1.06
GH + 10 mM gossypol	0.7	236.3	0.004	237	1.68

GH = grass hay, (a + b) is associated with potential gas production; c = the rate constant of (a + b); and a, b and c are constants. RSD = residual standard deviation.

Table 3. pH of supernatants and DM losses (g kg^{-1}) of GH with and without gossypol at 24 h of incubation (n = 2)

Substrates	DM losses (g kg^{-1})	pH
GH	804.24^{d} (800.04 - 808.44)	7.49 (7.48 - 7.49)
GH + 1 mM gp.	525.93^{c} (525.93 - 525.93)	7.30 (7.28 - 7.32)
GH + 5 mM gp.	465.62^{b} (465.61 - 465.63)	7.25 (7.20 - 7.30)
GH + 10 mM gp.	296.19^{a} (277.14 - 315.25)	7.38 (7.36 - 7.40)

GH = Grass hay, DM = dry matter. Means with different superscripts are significantly different (P<0.01, SED = 13.8). The values in the brackets are the two replicates values of the DM loss or pH.

Table 4. Volatile fatty acids concentration in the supernatant from grass hay with and without gossypol (n = 2)

Substrates	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
GH	18.86^{c} (18.33-19.39)	5.77^{a} (5.47-6.07)	8.16^{c} (8.13-8.20)
GH + 1 mM gp.	16.30^{cb} (15.97-16.63)	16.98^{b} (14.65-19.31)	8.48^{c} (8.31-8.66)
GH + 5 mM gp.	15.31^{a} (14.69-15.94)	17.76^{b} (16.53-18.99)	5.82^{b} (5.67-5.97)
GH + 10 mM gp.	13.77^{ab} (12.85-14.69)	18.05^{b} (16.53-19.57)	5.11^{a} (5.12-5.11)

GH = grass hay; 1mM gp.= 1mM gossypol concentration in culture. Means with different superscripts in the same columns are significantly different (P<0.05, SED = 2.5 for Acetic acid; P<0.05, SED = 6.05 for Propionic acid, and p<0.01, SED = 0.18 for Butyric acid). Values in the brackets are the two replicates values of the acetic, propionic and butyric acids.

The results in Table 2 shows that the rate of gas production (c) from GH is higher than that of GH treated with gossypol. However, the potential gas production (a + b) and the asymptote (b) of GH were much lower than gossypol treated substrates suggesting that the presence of gossypol in the culture resulted in more gas from GH. The reason for this is uncertain. However, the increase in gas production which occurred when gossypol was added to GH conversely correlated with the DM losses of the GH. In the presence of gossypol, there was a change in the proportions of VFA suggesting that the presence of gossypol may have altered the fermentative activity of rumen protozoa in culture. Gossypol significantly increased (P<0.05) the molar proportion of propionic acid and significantly decreased the molar proportion of acetic acid (P<0.05) and butyric acid (P<0.01). There was a good correlation between DM loss and acetic acid (0.9), and DM loss and butyric acid (0.76). A negative correlation (-0.87) was found between DM loss and the concentration of propionic acid.

Microscopic observation of the rumen fluid from syringes after 24 h incubation indicated that syringe containing the grass hay substrate has a very active population of rumen ciliates with very few dead organism. There were large number of *Isotricha* and *Ophrioscolex*, moderate number of *Dasytricha* and *Diploplastron* and a few *Entodinia* (both large and small) and one or two *Polyplastron*. The organisms were quite dark and full of starch, particularly the *Isotricha*. A number of hay particles were found in the sample. The addition of 1 mM gossypol resulted in the death of a lot protozoa, with only a few *Isotricha* and *Dasytricha* and one or two *Polyplastron* and *Ophryoscolex* alive. Gossypol (5 mM) killed

almost all of the protozoa, with only 4 to 6 *Isotricha* and two *Dasytricha* remaining alive in a typical field of view. The addition of 10 mM gossypol resulted in the presence only a very small numbers of *Isotricha* alive and no other live protozoa. It was concluded that gossypol was responsible for the death of about 50 - 75 % of rumen ciliates in comparison with the numbers of live ciliates in the hay substrate samples.

The increase in gas production of gossypol treated samples did not correlate with the effect of gossypol on the rates of gas production. However, the death of the major proportion of rumen ciliates may have contributed to the increase in gas production in the gossypol treated substrates. The H_2, CO_2 and CH_4 produce during fermentation (Wolin and Miller, 1983), may have been supplemented by CO_2 produced during the breakdown and/or lysis of the dead rumen ciliates.

Theodorou *et al.* (1995) found that accumulation of gas production from growth of the rumen fungi *Neocallimastix hurleyensis* and *Cacomyces communis* on glucose and cellulose carbon sources did not go hand in hand with biomass accumulation in older cultures and fungi that stopped growing continued to produce gas. The rumen fungi and possibly also protozoa may, despite the cessation of growth, continue to show fermentative activity, or they may release gas on physical rupture, or they may acidify the medium and thus release CO_2 from solution. It has been reported that additional gas could also be released from dissolved HCO_3 salts by volatile fatty acids (O'Hara *et al*, 1974; Menke and Steingass, 1988). All these factors may contribute to the additional gas production during fermentation. These factors may also explain the high gas production from grass hay treated with gossypol in the present experiment.

Conclusion

The results of this experiment shows that the rate of gas production (c) from grass hay (GH) is higher than that of GH treated with gossypol. However, the potential gas production (a + b) and the asymptote (b) of GH were much lower than gossypol treated substrates. It was suggested that the presence of gossypol in the culture resulted in more gas from GH. The reason for this is uncertain. However, the increase in gas production which occurred when gossypol was added to GH conversely correlated with the dry matter (DM) losses of the GH.

References

Eadie, J. M. and A. E. Oxford. 1955. Factors involved in the production of a novel kind of derangement of storage mechanism in living holotrich ciliate protozoa from sheep rumen. J. Gen. Microbiol., 12: 198-310.

Ismartoyo, T. Acamovic, and C. S. Stewart. 1993. The effect of gossypol on the rumen microbial degradation of grass hay under consecutive batch culture (CBC). Anim. Prod., 56: (Suppl.1).462 (A.).

Ismartoyo, C. S. Stewart, W. J. Shand and T. Acamovic. 1994. The effect of gossypol on the rumen protozoal degradation of grass hay (GH) in vitro. VIII International Symposium on ruminant Physiology. 25-30 September 1994, Willingen, Germany. p. 205.(A).

Ismartoyo, T. Acamovic, C. S. Stewart, A. J. Richardson, S. H. Duncan and W. J. Shand. 1995. The effect of feeding WCS (whole cottonseed) as a supplement for sheep fed GH (grass hay) on the rumen fermentation and defaunation of rumen microorganism. Conference on the Evaluation of Tropical Forages for Ruminant Utilisation. Held in University of

Zimbabwe, August 27 to September 1st, 1995.

Ismartoyo. 1999. Analysis of gossypol in whole cottonseed and rumen liquor of sheep fed a basal diet grass hay supplemented with whole cottonseed. Buletin Ilmu Peternakan dan Perikanan. Vol. V (13), April 1999. p.11-31.

Menke, K. H. and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Develop., 28: 7-55.

O'Hara, M., S. Hozumi, and K. Ohki. 1974. Studies on the mode of gas production in an artificial rumen and its application to the evaluation of feedstuffs. IV. On the role of biocarbonate buffer for gas production. Jap. J. Zootechn. Sci., 45: 1-7.

Ørskov, E. R. and E. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate passage. J. Agric. Sci. (Cambridge.), 92: 499-503.

Risco, C. A. and C. C. Chase, Jamie Robertson. 1997. Gossypol. In: Plant and fungal toxicants. (Ed. J.P.F. D'Mello). CRC Press, Boca Raton, Florida. pp. 243-252.

Theodorou, M. K., D. J. Gassoyne, D. E. Akin and R. D. Hantley. 1995. Effect of phenolic acids and phenolics from plant cell walls on rumen like fermentation in consecutive batch culture. Appl. Environ. Microbiol., 53:1046-1050.

Wolin, M. J. and T. L. Miller. 1983. Carbohydrate fermentation. In: Human intestinal microflora in health and disease. (Ed. D. J. Hentges). Academic Press, Inc. pp. 147-165.